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Chia protein hydrolysates: Characterization and emulsifying properties

Running title: Emulsifying properties of chia hydrolysates

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Abstract

The evaluation of functional properties of different chia protein hydrolysates (CPH) and their application in O/W emulsions were studied. Enzymatic treatments with pepsin, pancreatin, or the sequential action of pepsin-pancreatin were applied to hydrolyse a chia protein concentrate (CPC). Oil-in-water emulsions stabilized with CPC or these CPHs, with or without chia mucilage, were prepared at pH 7 or 10. Particle size, global stability, ζ-potential, and rheological measurement of emulsions were determined. CPH presented higher ($p \leq 0.05$) solubility and surface hydrophobicity levels, exhibiting better emulsifying properties than CPC. Emulsions with CPH presented smaller ($p \leq 0.05$) droplet sizes than those with CPC. Regarding to physicochemical stability, emulsions at pH 7 were less stable than those at pH 10, showing destabilization by creaming and coalescence. The addition of chia mucilage increased the apparent viscosity of emulsions and led to modifications in their fluid behaviour, exhibiting an interesting role as a thickening agent.

Keywords: *chia protein concentrate; chia protein hydrolysates; chia mucilage; O/W emulsions; functional properties*

Introduction

There is a growing interest from academic and industrial sectors to find sustainable, natural, and minimally processed food ingredients that also present technological and functional potential. Plants are the most important natural sources of bioactive components to be applied in the development of functional foods. Among them, chia (*Salvia hispanica* L.) become increasingly important for human health and nutrition as a source of bioactive components in the last decades, being the highest vegetable source of omega-3 fatty acids (ω-3 FAs) known today. This oilseed has been considered a food by the US Food and Drug Administration (US Food and Drug Administration, 2009), and the European Parliament and European Council have approved it as a novel food (European Commission, 2009) (Julio et al. 2018). On average, this seed contains ~33% of oil rich in essential omega-3 polyunsaturated fatty acids (ω-3 PUFAs), with ~60% in the form of α-linolenic fatty acid. The consumption of adequate levels of these ω-3 essential lipids is associated with multiple beneficial effects on human health (Lorente-Cebrián et al. 2013). Besides, chia has appreciable levels of dietary fibre and proteins, with contents of ~ 30 and 19-23%, respectively (Julio et al. 2016). Another relevant feature of chia seed is that it does not contain gluten (Julio et al. 2018).

The industrial chia oil production is carried out mainly through the cold press extraction with the consequent generation of considerable amounts of a defatted food. Despite these industrial by-products have appreciable fibre and protein contents, they are currently intended to produce low-cost products, such as partially defatted flour or animal feed. Thus, the possible obtention of chia mucilage and proteins of high biological and nutritional values from this by-product of the oil industry has gained attention as a strategy to minimize this waste disposal and diversify the offer of products made with high added value (Cotabarren et al. 2019). Some research works have studied the chia mucilage extraction through different methodologies (Capitani et al. 2013; Muñoz et al. 2013; Orifici et al. 2018). On the other hand, the research by Sandoval-Oliveros and Paredes-López 2013 has proven that chia proteins present high nutritional quality due to their have good levels of digestibility (~78.9%) and also all the essential amino acids for human nutrition. Julio et al. (2019), López et al. (2018) and Martínez et al. (2017) have isolated and characterized chia proteins, the latter reported that the major fractions are globulins, followed by albumins, glutelins and in a lower proportion prolamins with 39.2, 27.6, 25.8 and 7.3 g/100 g of seed, respectively. In the electrophoretic pattern, they observed that albumins had low intensity bands between 15-220 kDa, globulins and prolamins intensity bands between 18-35 and 15-19 kDa, and glutelins with globulins-like bands.

The consumer trend towards vegetarianism and the increasing demand for functional foods, pharmaceutical and cosmetic ingredients from vegetal sources have encouraged the production of purified protein derivates of vegetal origin, such as concentrates, isolates, and hydrolysates. Thus, an alternative for the revaluation of these chia by-products is their potential use as a source of bioactive peptides through their hydrolysis, which beyond adding value would allow their transformation into products with nutraceutical properties. In this regard, most of the works have employed enzymatic hydrolysis and have dealt with the study of the biological activities with benefits to human health of chia hydrolysates (Segura-Campos et al. 2013; Chim-Chi et al. 2018; Coelho et al. 2018), being scarce the knowledge available about their functional properties.

It is known that the hydrolysis process can affect the protein functional properties due to the reduction of their molecular weight, enhancement of polar groups, and modification of molecular configuration. Thus, the study of the solubility and emulsifying properties of chia hydrolysates is relevant to evaluate their potential application in the development of functional foods. In the case of emulsification widely utilized in the food industry to obtain products like mayonnaise, cream, sauces, desserts, comminuted meat products and some beverages the most important foods emulsifiers are proteins. Therefore, the technological uses of chia proteins hydrolysates depend largely on their functional and physicochemical properties, which are necessary for their successful incorporation into food systems.

The present research work aimed to obtain and characterize a chia protein concentrate (CPC) and different chia protein hydrolysates (CPHs), in terms of their solubility, degree of hydrolysis, and hydrophobicity. Chia oil-in-water emulsions (O/W) with CPC or CPHs, at different pH levels with or without chia mucilage were also obtained and characterized.

Materials and methods

Materials

Chia seeds and oil were purchased from a local market of Mérida city (Yucatán, México). All reagents used were of analytical grade and purchased from JT Baker (Phillipsburg, NJ, USA) and Sigma Chemical Company (St. Louis, MO, USA).

Chia seed by-products

The extraction of the chia mucilage (CM) was performed following the methodology proposed by Us-Medina et al. (2018). Briefly, a suspension 1:40 (wt/v) of whole chia seed in water was stirring for 2 h

at 60°C to promote the mucilage exudation. The suspension was cooled, filtered and, freeze-dried. The dried CM was stored in a desiccator until use.

A chia protein concentrate (CPC) was obtained according to Salazar-Vega et al. (2020). After the CM extraction, the residual seed material was ground with a laboratory Thomas-Wiley mill (Model 4, Thomas Scientific, USA) and subjected to lipid extraction with n-hexane. The defatted material was dried at 60°C for 12 h and then introduced in a Ro-Tap type agitation and sieving system for 20 min to obtain particle sizes smaller than 140 µm. The soluble protein was obtained by dispersing the powder material into distilled water (1:20 wt/v), adjusting the pH to 12, and mixing for 30min. The dispersion resulting was centrifuged for 60 min at 2512 *g* and the supernatant was collected. The pH of the supernatant was adjusted to 4, stirred for 30 min and finally centrifuged at 2512 *g* for 60 min. The precipitate was recovered and lyophilized. The powder obtained was stored into a desiccator until use.

The proximal chemical composition of the CM and CPC was determined according to official methods (Paez et al. 2016). Protein content was estimated as nitrogen x 6.25, and carbohydrate content was expressed as nitrogen-free extract (NFE). All determinations were made in triplicate.

Preparation of chia protein hydrolysates (CPH)

CPC hydrolysis was performed under controlled conditions using a reaction vessel containing a thermometer, stirrer, and pH electrode attached. CPC was suspended in distilled water to a concentration of 4% (wt/v), adjusting the optimal temperature and pH for each enzyme before their addition. Then, proteases were added at an enzyme: substrate ratio of 1:10 and incubation for 60 min heating at 37°C, at pH 2 and 7.5 for pepsin and pancreatin, respectively. Also, sequential hydrolysis was performed using the pepsin-pancreatin system. It consisted of a pre-digestion for 60 min with pepsin followed by incubation for 60 min with pancreatin. In all cases, reactions were stopped by heating at 80°C for 20 min, followed by centrifugation (Ultracentrifuge Beckman Coulter LE-80K, California, USA) at 9460 *g* for 30 min to remove the insoluble fraction. The three chia protein hydrolysates obtained from treatment with pepsin, pancreatin, and the sequential hydrolysis are named PEP, PAN, and SEC, respectively.

Degree of hydrolysis (% DH)

The degree of hydrolysis (%DH) was calculated according to Nielsen et al. (2001). Briefly, aliquots of 200 µL of samples aqueous dilutions were mixed with 1.5 mL of the o-phthaldialdehyde (OPA)

reagent. The mixtures were shaken and after 2 min of incubation the absorbance at 340 nm was measured. %DH determinations were made in triplicate and calculated from the following equation:

$$\%DH=h/h_{tot}*100$$

Where h_{tot} is the total number of peptide bonds per protein equivalent and *h* is the number of hydrolysed bonds that was calculated as follows:

$$h = (\text{Serine-}NH_2 - 0.4)$$

$$\text{Serine-}NH_2= OD_{sample} - OD_{blank}/OD_{standard} - OD_{blank}*0.9516\ m_{eqv}/L\ *0.1\ *\ 100/\ X\ *\ P$$

where serine-NH_2= m_{eqv} serine NH_2/g protein; X = g sample; P = protein % in sample; 0.1 is the sample volume in litres (L).

Solubility

The solubility of the CPH was performed according to Were et al. (1997). Briefly, 125 mg (d.b.) of each sample was suspended into 25 mL of distilled water adjusting the pH to 2, 4, 6, 8, 10, and 12 using NaOH or HCl 0.1N. Then, after stirring for 30 min in a magnetic stirrer, the dispersion was centrifuged at 3838 *g* for 45 min. The volume of the supernatant was measured and used to determine the nitrogen content by the method 954.01 of the AOAC (2016).

Surface Hydrophobicity

Hydrophobicity was determined according to Kato and Nakai (1980), using 1-anilinonaphthalene-8 sulfonic acid (ANS) as a probe. A series of solutions with concentrations from 0.05 to 0.5 mg/mL in buffer phosphate 0.1M were prepared. Afterwards, 2 mL of each sample solution was mixed with 30 µL of 8 mM ANS solution in the buffer. Finally, the fluorescence intensity was measured at 364 nm (excitation) and 484 nm (emission) using a Fluorescence Spectrometer (PerkinElmer LS-50B, Massachusetts, USA).

Oil-in-water (O/W) emulsions

Preparation of O/W emulsions

O/W emulsions consisting of 20% wt/wt of chia oil and 80% wt/wt of aqueous phase were prepared at two pH levels (7 and 10). For this purpose, different aqueous phases were prepared by dissolving 1.5% wt/wt of wherever CP or CPH (PEP, PAN, or SEC) and, 0 or 0.75% wt/wt of CM in distilled water. The oily and aqueous phases were homogenized at 13,500 rpm for 2 min using an Ultraturrax T-25 (Janke & Kunkel, IKA-Labortechnik, Germany) and treated for 1 min with an ultrasonic

processor VCX 750 (Sonics & Materials, Inc., USA) at 50% of the amplitude to reduce the droplet size. Nisine (0.0012g/100g) and potassium sorbate (0.1g/100g) were added to the emulsions to prevent microbial growth.

Particle size

The particle size of emulsions was determined using the static light scattering technique with a Malvern Mastersizer 2000E (Malvern Instruments Ltd., Worcestershire, UK). Each sample was suspended directly in a dispersion system at 2,000 rpm. The refractive indices used were 1.47 and 1.33 which correspond to the dispersed and the continuous phase, respectively. Three measurements were performed for each sample.

ζ-Potential

The electrical charge on the surface of the oil droplets was obtained at 25 ± 0.3 °C by measuring the electrophoretic mobility (μ) using a Zeta Potential Analyzer (Brookhaven 90Plus/Bi-MAS, USA) (Julio et al. 2015). The μ was converted to ζ-potential values using the Smoluchowski equation. Measurements were performed in quintuplicate.

Rheological properties

Rheological measurements were carried out at 25 ± 0.3°C using a controlled-effort rheometer (Haake RS6000, Thermo Electron Corporation, Germany) with concentric cylinders. The apparent viscosity (η) was recorded by increasing the shear rate ($\dot{\gamma}$) of 1-500 s^{-1} for 120 s, holding it at 500 s^{-1} for 60 s, and decreasing it from 500-1 s^{-1} for 120 s. Determinations were performed in triplicate.

Emulsion stability

The global stability of emulsions was evaluated using a Vertical Scan Analyzer (Quick Scan, Coulter Corp., Miami, USA). The emulsions were transferred into cylindrical glass tubes and scanned from the bottom to the top (~65 mm) with a near-infrared light source (λ=850 nm) acquiring the light backscattered (%) through the sample during 1,000 h.

Statistical analysis

The experimental results were analyzed by multifactorial ANOVA ($p \leq 0.05$) to study the main effect and the interactions between them, using the Statgraphics Centurion XV.II program for Windows

software (StatPoint Technologies, Warrenton, USA). Multiple comparisons between means were performed using the Tukey test ($p \leq 0.05$) (95% level of confidence).

Results and discussion

Chia seed by-products

The CM presented 8.8±0.5% of moisture and its proximal composition on dry weight basis (% d. b.) resulted in 10.2±0.3%, 5.6±0.1%, 17.23±0.3%, 1.5±0.4%, 65.4±0.6%
209 for ash, protein, crude fibre, lipid, and nitrogen-free extract (NFE) contents, respectively. Additionally, the CPC content of moisture, ash, protein, crude fibre, lipid and NFE were 3.4±0.1%, 2.3±0.0%, 83.6±0.6%, 1.2±0.1%, 0.4±0.0% and 12.4±0.4%, respectively. Chim-Chi et al. (2018) reported lower values of protein content (77.26% d. b.) in a protein concentrate obtained by a similar method.

The DH for chia hydrolysates PEP, PAN and SEC were 22.8, 47.0 and 51.1%, respectively. The differences found in this parameter could be due to the enzymes used since they have different sites of action to break peptide bonds. In the case of pepsin, it hydrolyses the C-terminal end of residual aromatic amino acids, such as Tyr and Phe. These amino acids are present in the whole chia grain in amounts of 6.10 and 11.61 g/kg, respectively. On the other hand, pancreatin is a protease that includes the enzymes trypsin, chymotrypsin, and elastase. They are considered serine-proteases and have in their active site the amino acid serine (Sosa et al., 2018). DH results derived from the hydrolysis with pepsin-pancreatin were higher than those reported by Chan-Zapata et al. (2019) (38.31%) in *S. hispanica* employing the same sequential enzymatic system but with shorter hydrolysis time (45 min).

Protein solubility is considered a critical property of protein hydrolysates because it determines its potential application to protein-based or protein-fortified food products.

Functional properties of proteins, such as emulsifying, foaming, and gelling capacities, are also greatly influenced by their solubility, which in turn is dominated by the pH (Guo et al. 2019). **Fig. 1** shows the pH-dependent solubility profiles of CPC and the different CPH. According to the multifactorial ANOVA, both the chia protein type and the pH significantly affected the solubility (data not shown). Overall, the lowest solubility values were found at pH 4, which would be associated with the isoelectric point (pI) of chia proteins reported to be near pH 3 (Olivos-Lugo et al. 2010). Proteins solubility increase when the pH is above or below their pI because electrostatic repulsion interactions are greater than the hydrophobic ones (Zayas and Zayas 1997). Thus, the low solubility of chia

proteins in acidic medium was improving significantly as a function of the increase of pH. At a pH greater than 4, the solubility of CPC and CPH increased to reach the maximum value at pH 12. Similarly, Timilsena et al. (2016) found the minimum and maximum levels of chia protein solubility at pH 3 and 12, respectively. In the range of pH 2-10, all CPH were more soluble ($p \leq 0.05$) than CPC denoting an enhancement of this property after the hydrolysis process. A similar trend was found by Urbizo-Reyes et al. (2019), who observed that the solubility was higher for hydrolysates than the non-hydrolysed chia control protein in a pH range of 3-9. Furthermore, the major solubility at pH 10-12 was exhibited by SEC hydrolysates, which in turn, presented the highest %DH. The improvement of the CPH solubility would be due to their smaller molecular size in comparison to the intact protein. In this sense Sosa et al. (2018) hydrolysed a chia protein-rich fraction through a sequential system using pepsin-pancreatin, subsequently ultrafiltered the hydrolysate and quantified the protein content, they found that hydrolysate was composed of low molecular weight peptide fractions, being the fraction of 5-10 kDa and that of more than 10 kDa those that contained the highest amount of protein with 0.909 and 0.938 mg /mL, respectively.

Surface hydrophobicity (S_0) is a property indicative of the percentage of hydrophobic amino acids exposed on the surface of proteins. Therefore, S_0 is related to solubility, occurrence of aggregation or denaturation and exposure of hydrophobic components from protein structure. The S_0 corresponding to CPC, SEC, PEP, and PAN hydrolysates were 0.624, 1.856, 3.628, 3.748, respectively. Since CPH presented high S_0 values, it might suggest that hydrophobic residues immersed inside the protein molecule gradually appeared on the molecular surface as a result of the unfolding of the protein, which may explain the increasing protein-lipid and protein-protein interactions. According to Benítez et al. (2008), the protein hydrolysis modifies their molecular properties as a result of the molecular weight decrease, charge increase and release of hydrophobic groups, among other changes. The more hydrophobic a protein is, the lower its interfacial tension and the greater its emulsifying activity; that is, the most hydrophobic proteins more easily orient their non-polar groups towards the fatty phase by interacting more quickly with it.

Emulsifying properties

In order to evaluate the emulsifying properties of the CPC and the CPH, chia O/W emulsions were prepared at pH 7 and 10 in presence or absence of CM.

Droplet size is one of the main parameters in emulsions characterization. Generally, smaller droplet sizes are associated with higher physical stability of emulsifying systems. Thus, it is important to

achieve small droplet sizes during the homogenization process to avoid the emulsion destabilization by creaming or flocculation. Urbizo-Reyes et al. (2019) hydrolysed chia protein using enzymatic hydrolysis and found that the oil-droplet-size in hydrolysed proteins was smaller, increasing the emulsion capacity of the unhydrolyzed protein. They attributed this to a vast exposure of hydrophobic residues and the presence of electrostatic repulsion among droplets.

According to the multifactorial ANOVA, the pH (A), chia protein type (B), and the CM addition (C) had significant effects on the droplet mean diameter ($D_{4.3}$) and Span in this order (**Table 1**). Also, all the interactions between factors were significant, being the pH the factor with major influence.

The particle size distribution (PSD) of systems at pH 7 and 10, either with or without CM, is displayed in **Fig. 2.** As can be seen, the curves corresponding to emulsions at pH 7 presented ranges of particle sizes higher ($p \leq 0.05$) than those prepared at pH 10. Furthermore, although curves at pH 7 were narrower than those at pH 10, these were shifted to the right (**Table 2**). This tendency was observed both in all the emulsions studied and after the CM addition. Similarly, at pH 7 larger ($p \leq 0.05$) droplet sizes (17.490 to 177.585 μm) were recorded in comparison with those at pH 10. Particle sizes obtained (1.003 -13.065 μm) at this latter pH level could be attributed to the higher solubility of the systems at alkaline medium. In this condition, there is more chia protein available to act as an emulsifying agent and to be adsorbed into the droplet interfaces.

CPC emulsions presented larger particle sizes ($p \leq 0.05$) than those with hydrolysed proteins, significantly noticeable at pH 7. On the other hand, SEC and PAN emulsions recorded the lowest droplet sizes. This behaviour is consistent with the solubility results previously discussed. The addition of CM had a slight influence on the emulsion droplet size. Systems with CPC and CM presented a significant increase in their particle sizes.

The ζ-potential, highly dependent on the pH and absolute value associated with emulsions stability, is a parameter indicative of the electrostatic charges at the surface of the droplets of O/W emulsions. Regarding this, highly charged oil droplets (> -30 mV or <-30 mV) would stabilize electrostatically. In contrast, systems with low ζ-potential have greater tendency to coagulate or flocculate (Wang et al. 2011).

As can be seen in **Table 1,** the pH was the factor with the major influence on the ζ-potential, followed by the chia protein type (CPC, PAN, PEP, SEC) and the CM addition to a minor extent. Likewise, all the factor interactions were statistically significant.

In accordance with **Table 2**, all systems at both pH levels studied presented droplets negatively charged. It was expected since most of the vegetable proteins at pH above their pI are negatively

charged. At neutral medium, the droplet charges ranged from -27.9 to -59.8 mV while at pH 10 those resulted between -41.9 and -99.3 mV. Timilsena et al. (2016) measured the ζ-potential of a chia seed protein isolate as a function of pH, recording values of ~-38 and ~-47 mV at pH 7 and 10, respectively. Additionally, Julio et al. (2016) reported ζ-potential of ~-23 mV from oil droplets coated by a chia protein-rich fraction.

It could also be observed that systems with CPC had droplets with charge lesser negative than those with CPH, which was more noticeable at pH 10. This fact could be explained by the hydrolysis of the native protein resulting in peptides with a major number of ionized groups.

The addition of CM into the emulsions led to a reduction ($p \leq 0.05$) of the absolute value of ζ-potential of the droplets, which was more significant at pH 10. A possible explanation for this fact could be the possible charge suppression by electrostatic interactions between polypeptide chains and charged groups of the CM which is a water-soluble anionic heteropolysaccharide composed of β-d-xylose, α-d-glucose, and 4-O-methyl-α-d-glucuronic acid in a ratio of 2:1:1, respectively (Lin et al. 1994). These results are in agreement with those reported by Julio et al. (2016). They indicated that the net charge of the oil droplets became significantly less negative when CM was incorporated in emulsions.

The rheological properties of O/W emulsions were mainly affected by the CM addition and a minor extent by both the chia protein type and the pH (**Table 1**).

Some typical processes such as flowing through a pipe, stirring or mastication are associated with apparent viscosities at 100 s^{-1} of shear rate (η_{100}) (McClements 2005). Therefore, the η_{100} values of the different systems studied are shown in **Table 2.** A remarkable increase ($p \leq 0.05$) of emulsions apparent viscosity was evidenced after the CM addition. Regardless of the pH level, the η_{100} ranged between 0.003-0.017 Pa.s and 0.061-0.163 Pa.s for emulsions prepared without and with CM, respectively. This significant effect of CM on the viscosity of emulsions could be due to its ability to produce high-viscosity dispersions at low concentrations (Capitani et al. 2013; Muñoz et al. 2013; Orifici et al. 2018). In this sense, CM can act as a thickening agent contributing to the stabilization of emulsions through the gel formation or continuous phase thickening, slowing down the movement of droplets due to gravity or Brownian motion.

Regardless of the CM addition, among the different emulsions, those with CPC and CM showed the highest value of η_{100} at pH 10. Besides, systems stabilized with the different CPHs presented higher η_{100} at neutral chemical medium than pH 10, especially noticeable with the CM addition.

The power-law model was applied to fit the experimental results and to obtain the consistency index (K) and flow behaviour index (n) (**Table 2**)**.** Most of the systems without CM at the two-pH studied

behaved as Newtonian fluids with values of n≈1. Only emulsions with PAN and PEP at neutral pH presented pseudoplastic behaviour with n<1. However, CM addition to emulsions led to changes in their fluid behaviour. Thus, all Newtonian systems became pseudoplastic fluids.

The global stability of emulsions was monitored through their optical characterization to spot signs and kinetics of destabilization processes such as creaming or flocculation.

Backscattering (BS) profiles of samples (%BS vs. tube length) were acquired periodically during 1,000 h and their changes over time at Zone I (bottom of the sample tube) and Zone II (top of the sample tube) were studied (**Fig. 3**). Changes on the %BS in the zone I of the sample tube may suggest destabilization by creaming, while those recorded in zone II could be associated with the stability of the cream phase formed **(Fig. 3a).**

As the graphs show, emulsions global stability was influenced by the pH being more evident in systems without CM. At pH 7, the BS initial (BS_0) was ~60-65 % with exception of PEP emulsions without CM which presented a BS_0 of ~44%. Conversely, the BS_0 corresponding to emulsions at pH 10 recorded BS_0 ~70-75% values higher than the previous ones, showing increased backscattered light. Since the BS_0 of the emulsions is directly dependent on the mean droplet diameter, higher BS values at pH 10 are consistent with to the smaller particle sizes at pH 7 (Mengual et al. 1999).

Systems without CM exhibited destabilization by creaming and, in some cases, coalescence processes. At neutral pH, the reduction of the %BS at the bottom of the sample tube (Zone I) as a function of the time was indicative of the creaming occurrence. Emulsions with SEC and CPC recorded a significant reduction of the %BS at 150 h while PAN systems did so at 6 h. Emulsions with PEP presented poor stability exhibiting creaming immediately after their preparation **(Fig. 3b, c).**

When emulsions were prepared at pH 10, the creaming process was slower, and to a lesser extent than at pH 7, only CPC systems recorded a significant decrease of %BS at 150 h. The reduction of the %BS as a function of time is caused by the migration of the oil droplets of the dispersed phase towards the upper zone of the sample tube. Oil droplets upward movement could lead to their concentration and thus to the appearance of a cream phase at the top of the sample tube (Zone II).

In turn, PEP and SEC emulsions at pH 7 recorded a decrease in %BS of Zone II as a function of time. This reduction may suggest destabilization by coalescence consisting of an increase in the droplet size and a decrease in the number of them. For emulsions at pH 10, there were no significant changes recorded in their %BS in Zone II as a function of time **(Fig. 3 g, i).**

In the case of emulsions with CM, all of them exhibited high global stability during the period examined. The %BS of these systems for both zones remained unchanged

(Fig. 3d, e, h, i). The important enhancement of emulsions stability as a result of the CM addition shows its potential role as a food thickener agent. Besides, these results are in agreement with those previously discussed. Thus, emulsions with smaller particle size and higher viscosities presented higher global stability.

Conclusions

Results showed that the hydrolysis of CPC through different enzymatic treatments (PAN, PEP and, SEC) enhances its functional properties. The three evaluated types of CPH recorded both higher solubility and surface hydrophobicity values compared to those of the CPC. Considering this, CPH proved to be suitable emulsifying agents for chia O/W emulsions, especially at pH 10. Emulsions with CPH presented droplets with smaller sizes and more negatively charged than those prepared with CPC.

Systems obtained at pH 7 were less stable than those at pH 10, recording destabilization by creaming and coalescence mechanisms. The incorporation of a moderate concentration of CM avoided emulsions destabilization for 1,000 h by slowing down the oil droplets movement. The addition of CM had a strong influence on the rheological properties of the systems by increasing their apparent viscosities and by changing their fluid behaviours.

This study provides valuable information on both CM extraction and different CPHs obtention from the waste of industrial chia oil production. Additionally, the incorporation of CPHs and CM into O/W emulsions was also studied. All CPHs exhibited suitable characteristics as emulsifying agents, while the CM presented attractive properties as a thickening agent. Therefore, CPHs and CM chia by-products, could be considered products with high-added value and potentially included in functional foods development.

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Data Availability Statement

Data that support our findings are available upon request to the authors.

Ethical Guidelines

Ethical approval was not required for this research.

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Annotated references

The references with asterisks are key because, among those cited and listed, these studies deal with important advances in chia hydrolysates.

Legends to Figures

Figure 1

Solubility of CPC and the different CPH (PEP, PAN, and SEC) as a function of the pH. Average values are shown (n=2).

Figure 2

Particle size distribution curves corresponding to CPC (a) and chia hydrolysates PEP (b), PAN (c), and SEC (d). Emulsions prepared at pH 10 with ▬ ▬ or without CM ▬▬ or pH 7 with ▬ ▬ and without CM ▬▬. Average values are shown (n=2). The coefficient of variation was lower than 1%.

Figure 3

Backscattering profile of emulsion as a function of the sample tube length and storage time at zones I and II (**a**). Destabilization kinetics of O/W emulsions at pH 7 (**b, c**) without CM, (**d, e**) with CM and at pH 10 without CM (**f, g**) and with CM (**h, i**). Average values are shown (n=2). The coefficient of variation was lower than 1%.

Table 1. Multifactorial analysis of variance (ANOVA) of physicochemical properties of chia O/W emulsions

Factor	Sum of square						
	Degrees of freedom	$D_{4.3}$	Span	ζ–potential	n	K	η_{100}
pH (A)	1	20617.7***	157.416***	7002.18***	0.05513***	0.1394*	0.00007*
Chia protein type (B)	3	17534.7***	37.3725***	2190.16***	0.05761***	5.7394***	0.00438***
Chia mucilage addition (C)	1	205.741***	9.56375***	367.88***	1.68926***	20.6801	0.05599***
AxB	3	613.19***	54.3215***	2345.57***	0.09866***	5.5753***	0.00422***
AxC	3	14061.2*	4.25299***	386.42***	0.05035***	0.1822*	0.00000
BxC	1	10.3968***	143.127***	329.197**	0.02985***	5.7993***	0.00410***
AxBxC	3	6348.47***	133.902***	305.61*	0.03022**	5.3666***	0.00327***
Pure error	16	1.56326	4.0721	220.476	0.00764	0.2728	0.00018
Total	31	1.179	544.028	13147.5	2.01874	43.7551	0.07224

* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$

Table 2. Droplet volume-mean diameter ($D_{4,3}$), Span, ζ-Potential, apparent viscosity at 100 s^{-1} (η_{100}), and rheological parameters (K, n) of chia O/W emulsions at pH 7 or 10 with or without the addition of chia mucilage.

pH	System	CM (% wt/wt)	$D_{4,3}$ (μm)	Span	ζ-Potential (mV)	K (Pa s^n)	n	η_{100} (Pa s)
7	PAN	0	18.687 ± 0.515 ^{e}	2.213 ± 0.021 ^{abc}	- 59.8 ± 4.5 ^{def}	0.026 ± 0.002 ^{a}	0.667 ± 0.014 ^{c}	0.006 ± 0.000 ^{ab}
		0.75	21.580 ± 0.099 ^{e}	1.503 ± 0.006 ^{ab}	- 42.8 ± 3.2 ^{bc}	1.807 ± 0.146 ^{e}	0.391 ± 0.016 ^{b}	0.107 ± 0.001 ^{g}
	PEP	0	81.900 ± 1.032 ^{g}	1.315 ± 0.064 ^{ab}	- 27.9 ± 4.3 ^{a}	0.063 ± 0.015 ^{a}	0.655 ± 0.016 ^{c}	0.017 ± 0.002 ^{b}
		0.75	18.735 ± 0.474 ^{e}	1.018 ± 0.007 ^{a}	- 37.6 ± 1.3 ^{abc}	1.329 ± 0.090 ^{de}	0.403 ± 0.003 ^{b}	0.077 ± 0.003 ^{de}
	SEC	0	27.940 ± 0.622 ^{f}	1.827 ± 0.021 ^{abc}	- 33.8 ± 1.3 ^{ab}	0.004 ± 0.001 ^{a}	0.967 ± 0.046 ^{e}	0.003 ± 0.000 ^{a}
		0.75	17.490 ± 0.071 ^{de}	1.535 ± 0.007 ^{ab}	- 46.4 ± 1.8 ^{bcd}	1.306 ± 0.259 ^{cde}	0.431 ± 0.034 ^{b}	0.086 ± 0.002 ^{ef}
	CPC	0	82.020 ± 1.541 ^{g}	2.070 ± 0.042 ^{abc}	- 43.0 ± 2.0 ^{bc}	0.008 ± 0.001 ^{a}	0.868 ± 0.031 ^{d}	0.006 ± 0.001 ^{ab}
		0.75	177.585 ± 4.320 ^{h}	1.915 ± 0.035 ^{abc}	- 38.3 ± 0.9 ^{abc}	1.538 ± 0.176 ^{e}	0.400 ± 0.013 ^{b}	0.095 ± 0.006 ^{f}
10	PAN	0	1.003 ± 0.007 ^{a}	3.592 ± 0.005 ^{e}	- 80.8 ± 7.5 ^{g}	0.003 ± 0.000 ^{a}	0.985 ± 0.004 ^{e}	0.003 ± 0.000 ^{a}
		0.75	2.945 ± 0.035 ^{a}	1.755 ± 0.007 ^{abc}	- 63.9 ± 4.4 ^{ef}	0.805 ± 0.067 ^{bcd}	0.460 ± 0.015 ^{b}	0.068 ± 0.005 ^{cd}
	PEP	0	1.220 ± 0.014 ^{a}	3.750 ± 0.0141 ^{c}	- 79.0 ± 2.3 ^{g}	0.003 ± 0.002 ^{a}	0.990 ± 0.002 ^{e}	0.003 ± 0.000 ^{a}
		0.75	8.985 ± 0.346 ^{bc}	13.930 ± 1.414 ^{e}	- 69.2 ± 4.3 ^{fg}	0.823 ± 0.075 ^{b}	0.449 ± 0.017 ^{b}	0.066 ± 0.001 ^{cd}
	SEC	0	5.030 ± 0.014 ^{ab}	7.980 ± 0.0283 ^{d}	- 99.3 ± 5.4 ^{h}	0.003 ± 0.000 ^{a}	0.973 ± 0.007 ^{e}	0.003 ± 0.000 ^{a}
		0.75	2.770 ± 0.014 ^{a}	1.840 ± 0.198 ^{abc}	- 80.7 ± 3.6 ^{g}	0.764 ± 0.048 ^{bc}	0.463 ± 0.013 ^{b}	0.061 ± 0.003 ^{c}
	CPC	0	4.787 ± 0.604 ^{ab}	2.430 ± 0.085 ^{abc}	- 51.6 ± 6.1 ^{cde}	0.014 ± 0.005 ^{a}	0.862 ± 0.023 ^{d}	0.009 ± 0.000 ^{ab}
		0.75	13.065 ± 1.124 ^{cd}	3.270 ± 0.127 ^{bc}	- 41.9 ± 7.5 ^{abc}	4.650 ± 0.313 ^{f}	0.274 ± 0.001 ^{a}	0.163 ± 0.010 ^{h}

Different letters indicate significant ($p \leq 0.05$) differences in the same column

Figure 1.

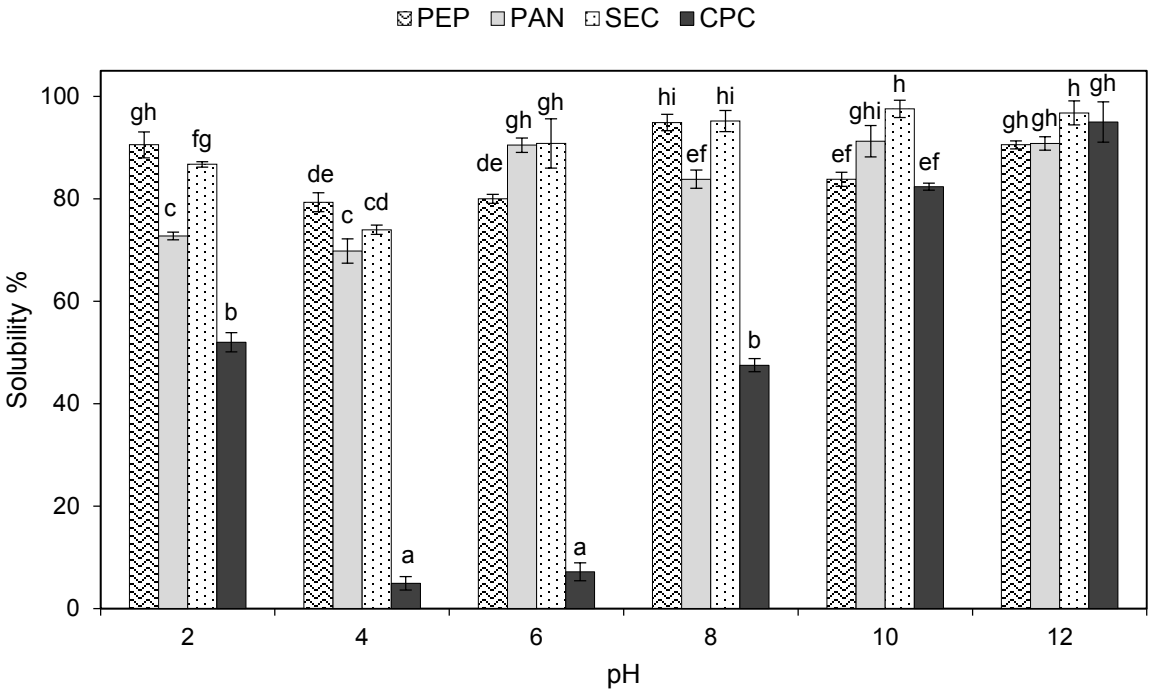

Figure 2.

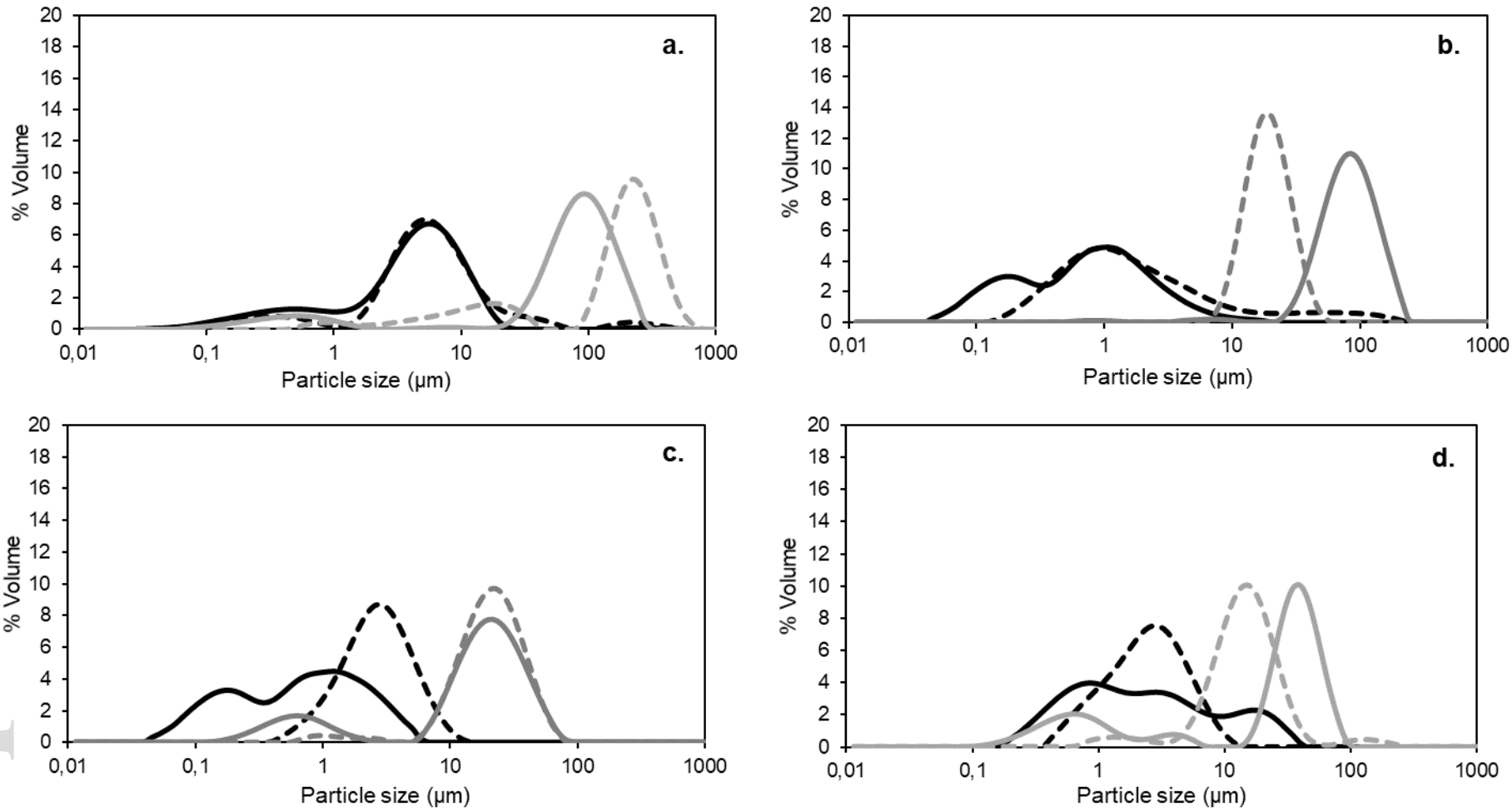

Figure 3.